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(54) **Title:** MUCIN-1 DERIVED ANTIGENS AND THEIR USE IN IMMUNOTHERAPY

(57) **Abstract:** Peptides and polypeptides capable of eliciting the immune response are disclosed which comprise an amino acid sequence substantially corresponding to that of an epitope of the non-VNTR, non-leader region of mucin. The peptides or polypeptides are useful themselves, or in the form of fusion proteins or conjugate compounds with carbohydrate polymers, in the prevention or treatment of disease states, particularly carcinomas such as adenocarcinomas. DNA vaccines and uses of the peptides and polypeptides for pulsing dentritic cells for *in vivo* transfer are also disclosed.

Mucin-1 derived antigens and their use in immunotherapy

Field of the Invention:

This invention relates to the immunotherapy of disease states, such as the immunotherapy of carcinomas.

Background of the Invention:

Cancer is a major cause of death and severe trauma in modern society. Cancer is not limited to one group; the young, old, males, females and peoples of all races may contract cancer, although cancer in children is relatively rare, perhaps with the exception of childhood leukemia. In western society, cancer of the colon and lung cancer are major diseases. In women, breast cancer is the most common form of cancer.

Many cancers are accompanied by overproduction of human mucin. Mucins are heavily glycosylated proteins (greater than about 100Kd) which are produced by many epithelial cells and tumours (1). Mucins found on cancer cells are different in some respects to those on normal epithelial cells, in that some mucins have a deficiency in their carbohydrate coat which leaves the protein core exposed (2). There are twelve forms of known human mucin designated MUC1 to MUC12 (see, for example, 3, 4, 26, 27). MUC1 is the most ubiquitous. The various mucins all have very similar properties, that is, they are transmembrane glycoproteins, all having a variable number of repeated amino acid sequences, which have a high content of serine, threonine and proline. Overproduction of aberrantly glycosylated mucins (either non-glycosylated or a deficiency in glycosylation) is characteristic of tumours of the breast, ovary, pancreas, colon, lungs, prostate and other tumours of secretory tissue. The cDNA sequences of the respective protein cores of the human mucins MUC1 to MUC7 have been cloned and characterised and have been found to contain highly repetitive central portions of varying numbers of repeats of particular amino acid motifs (known as VNTR's).

The surgery associated with tumour removal is traumatic to the patient, often disfiguring, and costly. Established chemotherapeutic and radiation procedures for tumour treatment which may be carried out in place of or in conjunction with surgical procedures are often debilitating and associated

with severe side-effects. There is, accordingly, an urgent need for therapeutic compounds and methods for the prevention/treatment of tumours.

Prior art relating to mucins mainly concerns the use of VNTR as a possible treatment or prophylactic for cancer. In one case, there has been a report of using the leader sequence of MUC1 to elicit cytotoxic T cells *in vitro* (71). However, studies involving this peptide, LLLLTVLTV (SEQ ID NO: 1) were conducted *in vitro* which is not necessarily indicative of how the peptide would behave *in vivo*. In addition, the epitope may not necessarily be a dominant T cell epitope of MUC1 and such lack of dominance may result in cytotoxic T lymphocytes (CTLs) to other epitopes, not LLLLTVLTV (SEQ ID NO:1).

In work leading to the present invention, the inventors surprisingly found that when a mannan-conjugate of HMFG (whole MUC1) was used for immunisation, non-VNTR, non-leader regions of MUC1 could be selectively antigenic. This is the first time that cytotoxic T cells to non-VNTR regions of MUC1 have been demonstrated in mice immunised with whole MUC1. This means that the non-VNTR peptides could have high affinity for the major histocompatability complex (MHC) class 1. This is surprising in view of the fact that VNTR peptides display low affinity for MHC class 1. The inventors' studies were conducted *in vivo*.

Disclosure of the Invention:

Accordingly, in the first aspect, the present invention provides a peptide or polypeptide capable of eliciting an immune response, wherein said peptide or polypeptide comprises an amino acid sequence substantially corresponding to that of an epitope of the non-VNTR, non-leader region of a mucin.

It is to be understood that the term "polypeptide" as used in the preceding paragraph and hereinafter does not encompass full-length mucin protein.

Preferably, the peptide or polypeptide consists entirely of an amino acid sequence derived from the non-VNTR, non-leader region of a mucin (and which includes an epitope). However, the peptide or polypeptide may include an additional amino acid sequence(s) derived from other regions of a mucin (including the VNTR and/or leader region). As such, the peptide or polypeptide may also include an epitope(s) from the VNTR and/or leader

region. Furthermore, the peptide or polypeptide may include an additional amino acid sequence(s) derived from other natural or artificial sources (e.g. the peptide or polypeptide may include a heterologous leader and/or signal sequence, or an amino acid sequence substantially corresponding to that of an epitope from an antigen from any tumour type or other source expressing MUC1). Examples of specific tumour antigens are carcinoembryonic antigen (CEA) from colon and other cancers or, indeed, antigens extracted from any tumour expressing MUC1.

Preferably, the immune response elicited by the peptide or polypeptide is a cell mediated immune response, particularly one involving the activation of cytotoxic T cells against cells expressing aberrantly glycosylated mucin (e.g. such as those characteristic of breast, overy, pancreas, colon, lung and prostate tumourigenic cells).

The term "substantially corresponding" as used herein in relation to amino acid sequences is intended to encompass minor variation(s) in the particular amino acid sequence which do/does not substantially alter the biological activity of the particular amino acid sequence. For example, in relation to the amino acid sequence of an epitope of a non-VNTR, non-leader region of a mucin, the term "substantially corresponding" encompasses variation(s) of that sequence (which variation(s) may be found in naturally-occurring variant sequences or otherwise) where the epitopic activity is substantially unaltered, i.e. the epitope variant is still capable of eliciting a substantially equivalent immune response. Such variations may include conservative amino acid substitutions. Conservative amino acid substitutions envisaged are:

G, A, V, I, L, M; D, E; N, Q: S, T; K, R, H; F, Y, W, H; and P, Nα-alkylamino acids.

The peptide or polypeptide according to the invention may be derived from natural sources, synthesised according to standard techniques or produced recombinantly. Peptide synthesis may be employed for polypeptides containing up to about a hundred amino acids. Generally, for polypeptides containing about twenty or more amino acids, the preferred means of production is recombinant expression in a host cell. Procedures for expression of recombinant proteins in prokaryotic and eukaryotic host cells are well established, see, for example, Sambrook, et al. (7).

The peptide or polypeptide may be part of a fusion protein. Procedures for expression of fusion proteins in prokaryotic and eukaryotic host cells are well established, see, for example, Sambrook, et al. (7). Fusion proteins may involve fusion of the peptide or polypeptide to a carrier protein selected from glutathione-S-transferase, β-galactosidase, or any other protein or part thereof, particularly those which enable affinity purification utilising the binding or other affinity characteristics of the protein to purify the resultant fusion protein. The fusion protein may involve fusion of the peptide or polypeptide according to the invention to the C-terminal or N-terminal of the carrier protein. The exact nature of the fusion protein will depend upon the vector system in which the fusion protein is produced. An example of a bacterial expression vector is pGEX which can be used to produce a fusion protein consisting of glutathione-S-transferase with a peptide, polypeptide or protein of interest. The carrier protein may or may not be cleaved from the peptide or polypeptide of the invention following expression. The fusion protein may be treated with mild periodate oxidation.

As mentioned above, expression of the peptide or polypeptide, or a fusion protein comprising the peptide or polypeptide, may be achieved using a host cell, e.g. a prokaryotic (e.g. *E.coli* or *B. subtilis*) or eukaryotic (baculovirus, CHO cells, COS cells or yeast) host cell expression system. In some of these systems, for example, baculovirus or yeast, glycosylation of the peptide, polypeptide or fusion protein can be achieved by introducing well known glycosylation motifs.

Similarly, the peptide or polypeptide may be simply coupled to a suitable carrier protein (e.g. keyhole limpet hemocyanin) using any of the well established procedures in the art (e.g. treatment with glutaraldehyde).

Preferably, the peptide or polypeptide according to the present invention comprises an amino acid sequence derived from human mucin 1. More preferably, the peptide or polypeptide comprises an amino acid sequence derived from human milk fat globule membrane antigen (HMFG). Even more preferably, the peptide or polypeptide comprises an amino acid sequence derived from the extracellular region or intracellular region of the non-leader, non-VNTR region of human MUC1 (e.g. amino acids 22 to 131, or amino acids 402 to 473 of human MUC1 according to NCBI database Accession No. M61170 (see also figure 1); although an amino acid sequence from the transmembrane region of the non-leader, non-VNTR region of

human MUC1 may also be suitable. Still more preferably, the peptide or polypeptide comprises an amino acid sequence substantially corresponding to one of the following amino acid sequences or an immunogenic fragment thereof:

TGSGHASSTPGGEKETSATQRSSVP	(SEQ ID NO: 2)
RSSVPSSTEKNAVSMTSSVL	(SEQ ID NO: 3)
SGHASSTPGGEKETSATQRSSVPSSTEKNAVSMTSSVLSSHSPGSGSSTTQG	
QDVTLAPATEPASGSAATW	(SEQ ID NO: 4)
SAPDNRPAL	(SEQ ID NO: 6)
NSSLEDPSTDYYQELQRDISE	(SEQ ID NO: 7)
TQFNQYKTEAASRVNL	(SEQ ID NO: 8)
AVCQCRRKNYGQLDIFPARDTYH	(SEQ ID NO: 9)
YVPPSSTDRSPYEKVSAGNG	(SEQ ID NO: 10)

In a second aspect, the present invention provides a compound comprising a conjugate of the peptide or polypeptide of the first aspect and a carbohydrate polymer.

Preferably, the carbohydrate polymer is a polymer of a carbohydrate selected from the group consisting of glucose, galactose, mannose, xylose, arabinose, fucose, glucosamine, galactosamine, rhamnose, 6-0-methyl1-D-galactose, 2-0-acetyl-β-D-xylose, N-acetyl-glucosamine, iduronate, guluronate, mannuronate, methyl galacturonate, α-D-galactopyranose 6-sulphate, fructose and α abequose, conformation and configuration isomers thereof, or a carbohydrate formed of two or more different monomer units. The number of repeated monomer units in the polymer is not important but generally carbohydrate polymers would comprise at least twenty monomer units, preferably in excess of one hundred monomer units, more preferably in excess of one thousand monomer units, and still more preferably in excess of ten thousand monomer units or more. Carbohydrate polymers may be a mixture of polysaccharide chains of varying molecular weights. More preferably, the carbohydrate polymer is a polymer of mannose or is a carbohydrate polymer containing mannose units. Most preferably, the carbohydrate polymer is a polymer of oxidised mannose.

The peptide or polypeptide according to the first aspect may be conjugated to a carbohydrate polymer according to standard techniques well known in the art of carbohydrate chemistry for the derivatization and reaction of polysaccharides and monosaccharides. Carbohydrates may be

oxidised with conventional oxidising reagents such as sodium periodate to give a polyaldehyde which can then be directly reacted with the peptide or polypeptide where amino functional groups on the peptide chain (such as the ε amino group of lysine) react with the aldehyde groups which may be further reduced to form a Schiff base. Polysaccharide chains may be first activated with cyanogen bromide and the activated polysaccharide then reacted with a diamine, followed by conjugation to the peptide or polypeptide to form a conjugate which may, optionally, then be oxidized. The carbohydrate and polypeptide may be derivatised with bifunctional agents in order to cross-link the carbohydrate and polypeptide. Commonly used cross-linking agents include 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicyclic acid, homobifunctional imidoesters including disuccinimidyl esters such as 3,3'-dithiobis(succinimidyl-propionate), and bifunctional maleimides such as bis-N-maleimido-1, 8-octane. Derivatizing agents such as a methyl-3-[(p-azido-phenyl)dithio] propioimidate yield photactivitable intermediates which are capable of forming cross-links in the presence of light. Oxidized carbohydrates may be reacted with hydrazine derivatives of antigens to give a conjugate. Alternatively, carbohydrates may be reacted with reagents such as carbonyl diimidazole followed by reaction with antigen, which after oxidation gives the desired conjugate.

The coupling of the peptide or polypeptide to a carbohydrate involves converting any or all of the functional groups on the carbohydrate to reactive groups and thereafter reacting the reactive groups on the carbohydrate with reactive groups on the polypeptide. Carbohydrate polymers are replete with hydroxide groups, and in some instances, carboxyl groups (such as in idruionate), ester groups (such as methylgalacturonate) and the like. These groups may be activated according to standard chemical procedures. For example, hydroxyl groups may be reacted with hydrogen halides, such as hydrogen iodide, hydrogen bromide and hydrogen chloride to give the reactive halogenated polysaccharide. Hydroxy groups may be activated with phosphorous trihalides, active metals (such as sodium ethoxide, aluminium isopropoxide and potassium tert-butoxide), or esterified (with groups such as tosyl chloride or acetic acid) to form reactive groups which can be then reacted with reactive groups on the polypeptide to form one or more bonds.

Other functional groups on carbohydrates apart from hydroxyl groups may be activated to give reactive groups according to standard techniques.

The carbohydrate polymer may be purified from a natural source or otherwise synthesised in accordance with standard techniques. Carbohydrates are available commercially from many suppliers.

The carbohydrate polymer is preferably conjugated to the peptide or polypeptide at any amount which permits the peptide or polypeptide to elicit a cell mediated immune response in a human or other animal. Such an amount may be within the range, for example, of about 0.1-10 mg per mg of the peptide or polypeptide.

Fusion proteins as described above and peptides or polypeptides otherwise coupled to a suitable carrier protein as described above, may also be coupled to a carbohydrate polymer (especially oxidised mannose). Similarly, the carbohydrate polymer is preferably conjugated to the fusion protein at any amount which permits the fusion protein to elicit a cell mediated immune response in a human or other animal. In this case however, the amount may be within the range, for example, of about 1-10 mg per mg of the fusion protein, more preferably about 5-8 mg per mg of the fusion protein.

In a third aspect, the present invention provides a vaccine against disease states, particularly human disease characterised by tumour cells expressing mucin or a subunit thereof, wherein said vaccine comprises the peptide or polypeptide of the first aspect of the invention, or a fusion protein comprising the peptide or polypeptide of the first aspect of the invention, and, optionally, an adjuvant and/or a pharmaceutically acceptable carrier.

In a fourth aspect, the present invention provides a vaccine against disease states, particularly human disease characterised by tumour cells expressing mucin or a subunit thereof, wherein said vaccine comprises the conjugate compound of the second aspect of the invention and, optionally, an adjuvant and/or a pharmaceutically acceptable carrier.

Suitable adjuvants for use in the vaccine of the third or fourth aspect include any of those well known in the art such as Quil A. QS-21 Iscoms, liposomes, alum, salts, oil, emulsions, etc.

The vaccine of the third or fourth aspect may be administered to human patients to protect against various disease states including cancer cell growth, and in particular, the growth of tumours of secretory tissues, such as

tumours of the breast, colon, lung, pancreas, prostate, and the like. Subjects may be immunised with the vaccine to protect against tumour formation of secretory tissues. Alternatively, subjects suffering from tumours may be immunised with the vaccine as part of a therapeutic regimen for tumour treatment. By way of example, to protect women from breast cancer, women may be immunised with the vaccine pre- or post-puberty and may receive one or more injections, preferably an initial immunisation followed by one or more booster injections separated by several months to several years. The route of immunisation is no different from conventional human vaccine administration. Accordingly, the vaccine of the third or fourth aspect may be administered subcutaneously, intramuscularly, orally, intravenously, and the like.

The amount of a peptide or polypeptide (which may be coupled to a suitable carrier protein), fusion protein or conjugate compound according to the present invention which is delivered to a subject is not critical or limiting. However, an effective amount of a peptide or polypeptide (which may be coupled to a suitable carrier protein), fusion protein or conjugate compound according to the present invention, is one which will stimulate an immune response. In this regard, the effective amount may vary according to the immune status of the subject (i.e. depending on whether the subject is immunosuppressed or immunostimulated), the judgement of the attending physician or veterinarian, whether the vaccine is to be used to prevent or treat a disease state or to prevent tumour formation, or whether the vaccine is to be used in the treatment of an existing tumour. By way of example, subjects may receive from 1μg to 10,000μg of the peptide or polypeptide (which may be coupled to a suitable carrier protein), fusion protein or conjugate compound according to the present invention, more preferably 50μg to 5,000μg, still more preferably 100μg to 1,000μg, and even more preferably 100μg to 500μg. Adjuvants are not generally required. However, adjuvants may be used for immunisation.

The peptide or polypeptide (which may be coupled to a suitable carrier protein), fusion protein or conjugate compound according to the present invention may be administered to subjects in concert with a cytokine or other immune regulator (e.g. one or more of GM-CSF, G-CSF, M-CSF, TNFα or β, interferon α or γ, any of IL1 through IL13, or any other cytokine). The immune regulator may be administered at the same or different time as the

peptide or polypeptide (which may be coupled to a suitable carrier protein), fusion protein or conjugate compound according to the present invention, optionally as part of a multi-component administration form.

In a fifth aspect, the present invention provides a method for inducing a cell mediated immune response against mucin which comprises administering to a subject an effective amount of the peptide or polypeptide of the first aspect (which may be coupled to a suitable carrier protein), or a fusion protein comprising the peptide or polypeptide of the first aspect, optionally in combination with an adjuvant and/or a pharmaceutically acceptable carrier.

In a sixth aspect, the present invention provides a method for inducing a cell mediated immune response against mucin which comprises administering to a subject an effective amount of a conjugate compound according to the second aspect, optionally in combination with an adjuvant and/or a pharmaceutically acceptable carrier.

The administration to human and animal subjects of the peptide or polypeptide (which may be coupled to a suitable carrier protein), fusion protein or conjugate compound according to the present invention may provoke a potentiated cellular response of activated T-lymphocytes which are cytotoxic to cells expressing mucins. A potential benefit of this invention arises from the fact that humans and animals may be protected against cancer prior to tumour growth, as the peptide or polypeptide (which may be coupled to a suitable carrier protein), fusion protein or conjugate compound according to the present invention of the invention may provoke a cellular immune response to cytotoxic T-cells which kill tumour cells expressing mucin. This invention is applicable to the immunisation against tumours of secretory tissue, such as adenocarcinomas, more particularly, tumours of the breast, ovary, pancreas, colon, lung, prostate and the like.

The peptide or polypeptide (which may be coupled to a suitable carrier protein), fusion protein or conjugate compound according to the present invention may also be used as, or as a component of, therapeutic agents for the treatment of patients suffering from cancer, as a part of the overall treatment for eradication or reduction of the cancer. Thus, the peptide or polypeptide (which may be coupled to a suitable carrier protein), fusion protein or conjugate compound according to the present invention may be administered to subjects suffering from cancer either before or after surgery to

remove a tumour. Preferably, the peptide or polypeptide (which may be coupled to a suitable carrier protein), fusion protein or conjugate compound according to the present invention is administered as part of a chemotherapeutic regime following tumour excision. In such circumstances, the peptide or polypeptide (which may be coupled to a suitable carrier protein), fusion protein or conjugate compound according to the present invention is administered in an amount consonant with standard chemotherapeutic regimes for the administration of cytotoxic compounds for use in tumour treatment.

It is believed that the peptide or polypeptide (which may be coupled to a suitable carrier protein), fusion protein and conjugate compound according to the present invention possess the advantage of being substantially non-toxic on administration to humans or animals, and as a consequence, are well tolerated.

In a further aspect, the present invention relates to the use of the peptide or polypeptide (which may be coupled to a suitable carrier protein), fusion protein or conjugate compound according to the present invention in the treatment of adenocarcinoma, particularly breast cancer.

In a still further aspect, the present invention relates to the use of the peptide or polypeptide (which may be coupled to a suitable carrier protein), fusion protein or conjugate compound according to the present invention to pulse dendritic cells for *in vivo* transfer and use as a vaccine.

In yet a still further aspect, the present invention provides an isolated nucleic acid molecule comprising a nucleotide sequence encoding the peptide or polypeptide of the first aspect (which may be coupled to a suitable carrier protein), or a fusion protein comprising the peptide or polypeptide of the first aspect.

The nucleic acid molecule may be incorporated into a transfer or expression vector, or used in a DNA vaccine. Such nucleic acid molecules may be produced according to standard techniques either by cloning or synthesis as described in, for example, Sambrook et al. (7).

In yet another aspect, the present invention provides a compound comprising a conjugate between MUC1 and a carbohydrate polymer such as those discussed above, such that the conjugate is capable of eliciting a cell mediated immune response in a human or other animal. Preferably, the MUC1 is human MUC1 (e.g. HMFG) and the carbohydrate polymer is a

polymer of mannose, particularly oxidised mannose, or is oxidised mannan. The carbohydrate polymer may be conjugated to the MUC1 at an amount within the range, for example, of about 1-10 mg per mg of MUC1, preferably about 5-8 mg per mg of MUC1, more preferably about 7 mg per mg of MUC1. The conjugate compound may be used in a vaccine or as a therapeutic agent in a manner akin to that discussed above.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia before the priority date of each claim of this application.

The invention will now be described with reference to the following non-limiting Examples and accompanying figures.

Brief description of the accompanying figures:

Figure 1: Provides the amino acid sequence of a human MUC1 protein (NCBI database Accession No. M61170).

Figure 2: Assay for HMFG and mannan. (a) Inhibition of binding of anti-MUC1 antibody to HMFG by competitor preparations of HMFG (m) and mannan-HMFG (l). (b) Binding of mannan-HMFG (l) and HMFG (m) to anti-MUC1 antibody and Con A detected by a radioimmunoassay.

Figure 3: A2K^{b}MUC1 double transgenic mice were immunised with mannan-HMFG and splenocytes were used in CTL assays. Cytotoxic activity of the effector cells were measured on ^{51}Cr-labelled MCF7 with (n) or without cold K562 (l); BT20 (p) or ME272 (m).

Figure 4: C57BL/6 and BALB/c mice were immunised with mannan-HMFG and splenocytes were used in CTL assays. Lysis of P815 (a) or RMA (c) cells pulsed with various 9-mer peptides from the intracellular peptide 471-493; Lysis of P815 (b) or RMA (d) cells pulsed with various 9-mer peptides

from the extracellular peptides 33-103 and 51-70 and (e) Lysis of P815 cells pulsed with YYQELQRDI (SEQ ID NO: 35) and RMA-MUC1 cells pulsed with SAPDNRPAL (SEQ ID NO: 36). As controls for peptide pulsing and antigen-specific cell lysis, known peptide antigens were used and are shown in each panel and described in the text.

Figure 5: Balb/c mice were immunised with mannan-507-KLH and splenocytes were used for CTL assays. The % lysis of ^{51}Cr-labelled P815 target cells unpulsed or pulsed with Cp13-32 or 507 peptide at various effector:target ratios were measured.

Figure 6: Balb/c mice were immunised with mannan-471-KLH and splenocytes were used for CTL assays. The % lysis of ^{51}Cr-labelled P815 target cells unpulsed or pulsed with Cp13-32 or 471 peptide at various effector:target ratios were measured.

Abbreviations:

The following abbreviations are used in the Examples:

ELISA: enzyme linked immunosorbent assay
DTH: delayed type hypersensitivity
FP: fusion protein
GST: glutathione-S-transferase
HMFG: human milk fat globule
Kd: kilodalton
KLH: keyhole-limpet haemocyanin
PAGE: polyacrylamide gel electrophoresis
PBS: phosphate buffered saline
SDS: sodium dodecyl sulphate
Tc: cytotoxic T-lymphocytes

EXAMPLE 1

1. INTRODUCTION

Immunotherapeutic approaches for the treatment of breast cancer have included the use of monoclonal antibodies and the generation of cytotoxic T lymphocytes (CTL) [29-34]. The identification of target antigens, the availability of recombinant proteins and cytokines have given impetus to immunotherapy. Thus, there are new means by which to generate an effective cytotoxic T cell response to MUC1-expressing carcinomas of the breast and

other tissues [35]. MUC1 is a particularly attractive target for the generation of CTL: it is immunogenic in mice for the production of antibodies and, more recently, the present inventors have described $CD8^+$ CTL, and the MHC Class I H-2 and HLA-A*0201 binding peptides have been mapped in the VNTR [36-39]. Furthermore, in cancer cells, there is up to a 100 fold increase in the amount of mucin [40] and there should be a significant amount of MUC1 peptide available to be bound by Class I molecules. The reason for the focus on the VNTR peptides is clear: it is the most immunogenic region in MUC1 when whole tumour cells or mucin extracts (HMFG) are used to immunise mice for the production of antibodies [40].

Because of this focus and the finding that non-HLA restricted CTL also are directed to the VNTR, almost all interest in MUC1 for CTL induction has concentrated on VNTR peptides [37, 41, 42]. In contrast, the present example relates to the induction of CTL to non-VNTR epitopes, in the extracellular and intracellular parts of MUC1 identified by immunising mice with native mucin (HMFG) obtained from human breast milk, or by immunising with peptides as described herein.

2. MATERIALS AND METHODS

Mice and Tumour Cells

BALB/c (H-2^d), C57BL/6 (H-2^b), human MUC1 transgenic mice (obtained from B. Acres (Transgene, Strasbourg, France)), transgenic HLA-A*0201/K^b mice (H-2^b)(obtained from The Scripps Clinic and Research Foundation, La Jolla, CA.) and double transgenic mice (A2K^bMUC1) were bred at The Austin Research Institute (ARI). The human MUC1 in the MUC1 transgenic mice (back crossed to DBA/2) is under the control of the human MUC1 promoter; MUC1 is expressed in the lung bronchioles, β-islets of the pancreas, kidney tubules and stomach [43]. The HLA-A*0201/K^b mice express a transgene composed of the α1 and α2 domains of HLA-A*0201 and the α3 contains the transmembrane and cytoplasmic domains of H-2K^b [44]. The double transgenic mice were screened for expression of the HLA-A*0201/K^b and human MUC1 transgenes by flow cytometry with antibodies to HLA-A*0201 and MUC1. RMA-MUC1 is a MUC1 transfected (C57BL/6 (H-2^b)) lymphoma cell line [45]. Tm211 is a MUC1 transfected P815 mastocytoma (DBA/2 origin; H-2^d) obtained from B. Acres (Transgene, Strasbourg, France) [46]. All mouse cell lines were maintained in Dulbecco's

modified Eagles medium (DMEM) with 100IU/ml penicillin, 100μg/ml Streptomycin and 10% foetal calf serum (all from Commonwealth Serum Laboratories (CSL), Melbourne) and human cell lines in RPMI with the same additives in a 7% humidified CO_2 incubator at 37°C. BALB/c, C57BL/6 and double transgenic A2K^{b}MUC1 mice were immunised intraperitoneally with 3 injections of 5μg mannan-HMFG or HMFG on days 0, 10, 17 while HLA-A*0201/K^b mice were injected once.

2.2 Synthetic Peptides

Peptides (Table 1) were synthesised at the ARI; the purity of the peptides (>95%) was determined by mass spectroscopy.

2.3 Conjugation of HMFG to Mannan

HMFG was isolated from human milk [49] and coupled to mannan. Mannan (1ml, 14mg/ml) in phosphate buffer (0.1M, pH6.0) was treated with sodium periodate (100μl, 0.1M) and incubated at 4°C for 30 min [48]. Ethanediol (10μl) was added for 30 mins at 4° to stop the reaction, and the mixture was passed through a PD10 column (Pharmacia Biotech, Sweden), equilibrated in bicarbonate buffer (0.2 M, pH 9.0) and the oxidised mannan fraction was mixed with 1 mg of HMFG overnight at room temperature to give mannan-HMFG.

2.4 T Cell Epitope Prediction

There are several CTL epitope prediction algorithms available and in this study we used the program developed by Dr Kenneth Parker available on the internet (bimas.dcrt.nih.gov/molbio/hla_bind/) to identify potential T cell epitopes. This program is based on scores given to the amino acids at each of the positions from 1-9 from input sequences by comparison with the reported databases [49, 50]. Higher numerical values for the 9-mer predict increased likelihood of being a T cell epitope. For example, the T cell epitope for ovalbumin (K^b, SIINFEKL; SEQ ID NO: 11) and papillomavirus-16 E7 protein (D^b, RAHYNIVTF; SEQ ID NO: 12) gives scores of 17 and 6 respectively.

2.5 Cytotoxic T Cell and Cytotoxic T Cell Precursor (CTLp) Frequency Assays

CTL assays were performed as described [37, 39, 48]. Briefly, 7 to 10 days after the final immunisation, splenocytes were harvested, washed and resuspended in growth medium and serially diluted in 96-well microtitre plates. A standard 3 hr ^{51}Cr release assay was performed with 1x10^4 peptide pulsed or untreated P815 or RMA cells as targets at various effector:target ratios. Peptide pulsed P815 or RMA target cells were prepared by overnight

incubation with 9-mer peptides (25μg/ml) [37] . For CTL assays with A2K^bMUC1 double transgenic effectors, MCF7 (MUC1$^+$HLA-A*0201$^+$) and BT20 (MUC1$^+$HLA-A*0201$^-$) breast cancer cell lines or the ME272 (MUC1$^-$ HLA-A*0201$^+$) melanoma cell line was used as targets. All of these human tumour cell lines are susceptible to cell mediated lysis [39, 51, 52]. CTLp frequencies were determined from a minimum of 32 replicates, for at least 6 effector cell numbers ($1x10^3$ - $1.28x10^5$). Cells were cultured in U-bottomed microtitre trays, with $5x10^5$ mitomycin C treated BALB/c (H-2^d), C57BL/6 (H-2^b) or HLA-A*0201/K^b spleen cells, in DMEM supplemented with 10% foetal calf serum, 5μM of various MUC1 peptides (Table 1) or HMFG and 10 U/ml rhIL-2. Seven days later, each microculture was assayed for cytotoxicity by replacing 100μl of culture medium with 100 μl target cell suspension containing 10^4 ^{51}Cr-labelled Tm211 (H-2^d), RMA-MUC1 (H-2^b) Tumour or EBV transformed human B cells (HLA-A*0201) or MCF7 as targets. As a specificity control non-MUC1 expressing P815(H-2^d) or RMA(H-2^b) cells were used. Cytotoxic activity was considered to be present if in each well ^{51}Cr release was found 3 standard deviations above the mean isotope release from 10^4 effectors cultured with stimulators only or from stimulator cells with peptide only or rhIL2 only. A linear relationship ($0.987 \leq r^2 \leq 1$) existed between the number of responder cells, represented on a linear scale, and the frequency of negative wells on a logarithmic scale. CTLp frequencies were determined as the inverse of responder cell dose required to generate 37% negative wells [53-55]. CTLp frequency assays were performed three times and the individual frequencies did not differ by more than 20% from the mean value. However, it should be noted that the CTLp frequency in immunised mice are directly correlated with tumour protection (28).

2.6 Inhibition ELISA

An antibody inhibition ELISA was performed to compare the activity of HMFG before and after conjugation to mannan. Polyvinyl chloride plates were coated with 70 μl of 10 μg/ml HMFG in bicarbonate buffer (0.2M, pH9.0) overnight at 4°C or 1 hr at 37°C and non-specific binding was blocked with 2% bovine serum albumin (BSA). Various concentrations of HMFG or mannan-HMFG were incubated with anti-MUC1 antibody (VA2 [57], 1/200 supernatant) for 3 hr and 100μl was added to PVC microtitre well plates coated with HMFG. After washing with phosphate buffered saline (PBS) containing 0.05% Tween 20, 50μl of sheep anti-mouse immunoglobulin

conjugated to horseradish peroxidase (Amersham, UK) was added and incubated for a further 1 hr at RT. After washing with PBS/Tween20, the plate was developed with the chromogenic substrate 2,2"-azino-di(3-ethylbenzthiazoline) sulphonate (ABTS) (Amersham, UK) and the absorption at 405 nm recorded.

2.7 Radioimmunoassay

A sandwich radioimmunoassay was performed to ascertain that the mannan was covalently linked to HMFG. A microtitre plate was coated with serial dilutions of anti-MUC1 antibody (BC2 [58]) in bicarbonate buffer overnight and non-specific binding blocked as described above. HMFG or Mannan-HMFG was then added to the wells and incubated for 1 hr at RT followed by washing extensively with PBS containing 0.05% Tween 20. Fifty μl of radiolabelled concanavalin A, which binds specifically to mannan but not HMFG, was then added and the plate incubated for a further 1 hr followed by washing with PBS/Tween 20. Microscint-O (120μl) was added to the wells, and plates counted in a β-scintillation counter.

3. RESULTS

3.1 Preparation and Characterisation of mannan-HMFG

The activity of the HMFG after conjugation to mannan was determined by inhibition ELISA; the 50% inhibitory concentration for HMFG was 22 μg/ml while for the mannan-HMFG was 20μg/ml (Fig. 1a), i.e. HMFG retained full reactivity after conjugation to mannan. The integrity of the mannan-HMFG complex was shown by a sandwich radioimmunoassay using anti-MUC1 antibody bound to the plate and ^{125}I-labelled Con-A for the read out (Fig 1b). Non-conjugated HMFG did not bind ^{125}I-Con-A while mannan-HMFG bound demonstrating mannan to be linked to HMFG.

3.2 CTL Responses to mannan-HMFG in BALB/c Mice

Spleen cells, from BALB/c mice immunised with mannan-HMFG, were stimulated *in vitro* with different peptides (from both VNTR and non-VNTR regions, Table 1) and CTLp were determined by testing on target cells expressing native MUC1 (Table 2). It was apparent that immunisation with mannan-HMFG leads to CTL reacting with epitopes from the whole of MUC1, i.e., from both the VNTR and non-VNTR region.

The responses were :-

a) HMFG. When whole MUC1 (HMFG) protein was used as the source of stimulating peptides, a CTLp frequency of 1/9,700 was obtained. Clearly HMFG is immunogenic for CTL production in BALB/c mice and can be processed to yield peptides presented by Class I molecules.

b) VNTR. When VNTR peptides Cp13-32 and p1-30 were used to stimulate, CTLp frequencies of 1/7000 (Cp13-32) and 1/13,200 (p1-30) resulted, i.e., by immunising with HMFG, anti-VNTR CTL were produced, results similar to those found previously by immunising with mannan-conjugated VNTR peptides [47]. This is the first description of such CTL obtained by immunising with native mucin which is glycosylated.

c) Extracellular regions. When *in vitro* stimulation was with peptides containing amino acids 31-55, 51-70, 33-103, 344-364, CTL could be detected with a frequency of 1/19,500 (31-55); 1/10,000 (51-70); 1/20,150 (33-103) and 1/36,800 (344-364). Thus, CTL can be produced to non-VNTR regions from the extracellular region; this is the first description of such CTL.

d) Intracellular regions. Three different, non-overlapping intracellular peptides containing amino acids 408-423, 471-493, 507-526, were examined using the approach described above. CTLp frequencies of 1/30,000 (408-423), 1/12,500 (471-493) and 1/22,500 (507-526) were obtained, amino acids 471-493 being the most effective to restimulate cytolytic cells.

To demonstrate that the CTL were specific for MUC1 sequences, and not due to non-specific killing by NK cells or other cells, P815 target cells were used with a non-MUC1 peptide, T4N1, as the pulsed antigen, CTLp either were not detected or the frequencies were ≤ 1/200,000 and were considered to be negative (not shown). Of the different regions, 3 were of equivalent immunogenicity (using CTLp frequency as a measure) : extracellular (51-70) = VNTR (Cp13-32) = intracellular (471-493), all of which gave a high frequency of ~ 1/10,000.

In contrast, immunising BALB/c mice with non-conjugated HMFG, and stimulating with the VNTR peptide Cp13-32, the CTLp frequency was 1/80,500. This frequency is similar to the CTLp frequency of 1/95,000 obtained with mannan conjugated to a recombinant bacterial fusion protein containing 5 repeats of the MUC1 VNTR (47) and, thus, conjugation of HMFG to mannan is necessary for generating a strong CTLp frequency in mice.

3.3 CTL Responses to mannan-HMFG in C57BL/6 mice

C57BL/6 were immunised with mannan-HMFG and *in vitro* stimulated with the same antigens used for the BALB/c mice (Table 2). There was a CTLp frequency of 1/13,500 for whole HMFG and 1/12,500 for the VNTR region peptide p1-30 (Table 2). Of the non-VNTR extracellular peptides, CTL were detected only to one extracellular peptide (344-364) with a frequency of 1/24,500. CTL were not detected to any of the intracellular peptides. Again, the specificity of the CTL were confirmed by using a non-MUC1 peptide, T4N1, for stimulation and also using the non-MUC1 transfected parent RMA cell line as the target. Thus, C57BL/6 mice can respond to both VNTR and non-VNTR peptides, but there were no responses to certain peptides to which BALB/c mice responded.

*3.4 Cellular Immune Responses to mannan-HMFG in Transgenic HLA-A*0201/K^b Mice*

Transgenic HLA-A*0201/K^b mice were immunised <u>once</u> with mannan-HMFG (not x3 as used above), stimulated *in vitro* with either HMFG, the VNTR peptide (p1-30) or one of the extracellular peptides (31-55). The CTLp were measured on human EBV HLA-A*0201$^+$ cells (see below) and frequencies were 1/39,000 (HMFG), and 1/33,000 (VNTR p1-30), which compare favourably with immunisation with mannan-VNTR peptide (1/48,000) i.e., whole HMFG is as immunogenic as VNTR (Table 2). Further, when an extracellular peptide (31-55) was used, the CTLp frequency was 1/40,000, i.e., the same as that found for VNTR. Thus, HLA-A*0201 can present extracellular and VNTR peptides. It should be noted that, the target cell being EBV transformed B cells, which expresses HLA-A*0201 but not H-2^b class I molecules (expressed by the immunised mice), the CTLs detected were restricted to HLA-A*0201 presenting MUC1 peptides.

3.5 Cellular Immune Responses to Mannan-HMFG in A2K^bMUC1 Double Transgenic Mice

To ascertain the ability of MUC1 CTL to lyse MUC1 positive breast cancer cells A2K^bMUC1 double transgenic mice injected with mannan HMFG 3 times were stimulated *in vitro* with either HMFG, the VNTR peptide (p1-30), extracellular peptides (31-55, 344-364) or intracellular peptides (408-423, 471-493, 507-526) (Table 2). There was a CTLp frequency of 1/2,000 for the whole HMFG and 1/8,000 for the VNTR region peptide p1-30. CTL were detected to the extracellular peptides 31-55 and 344-364 with a frequency of

1/2,000 and 1/11,000 respectively. Of the intracellular peptides, CTL were detected for only peptide 408-423 with a frequency of 1/20,000.

Spleens of the immunised mice were used in a direct CTL assay to ascertain specificity of the anti-MUC1 CTL. As seen in Figure 2, MUC1 CTL lysed 55% of MUC1$^+$ MCF7 (HLA-A*0201) breast carcinoma cells at an E:T ratio of 12:1 and was reduced to 17% when incubated in the presence of cold K562 targets. The MUC1 CTL were HLA restricted as no lysis was detected when the MUC1$^+$ BT20 (HLA-A1) breast cancer cell line was used. The MUC1 CTL did not lyse the MUC1 -ve melanoma cell line ME272.

Thus, immunisation of A2K^{b}MUC1 mice with mannan-HMFG resulted in specific Class I restricted CTL that can lyse tumour cells expressing native MUC1 and, moreover, anti-MUC1 CTL can be generated in mice in the presence of endogeneously expressed human MUC1.

3.6 T Cell Epitope Prediction and Mapping

To precisely map the T cell epitopes involved in CTLp generation, a large number of overlapping 9-mer peptides would have to be synthesised and used in CTL assays. Instead, a CTL epitope prediction program was used to select putative immunogenic peptides and these were synthesised to test their antigenicity.

Predicted H-2^d-restricted peptides (intracellular region MUC1)

Several peptides (NYGQLDIFP(K^d) SEQ ID NO: 13; YGQLDIFPA(D^d) SEQ ID NO: 14; KNYGQLDIF(L^d), SEQ ID NO: 15) were contained in 471-493 (CTLp frequency=1/12,500) and had predicted scores 6, 6 and 10 respectively (Table 3). To ascertain if the predicted 9-mers are presented by the Class I molecules, cytotoxic T cell assays were performed using spleen cells from mannan-HMFG immunised mice as effectors and P815 target cells were pulsed with the synthetic peptides. These were NYGQLDIFP(K^d) (SEQ ID NO: 13), YGQLDIFPA(D^d) (SEQ ID NO: 14), KNYGQLDIF(L^d) (SEQ ID NO: 15). The pulsed cells were not lysed by Mannan-HMFG derived CTL from BALB/c mice (Figure 3a), i.e., the CTL epitopes were not predicted accurately by the algorithm. The MUC1 VNTR peptides SAPDTRPAP(D^d) (SEQ ID NO: 16) and APDTRPAPG (L^d) (SEQ ID NO: 17) identified previously as CTL epitopes in the VNTR region [38], were used as positive controls and 62% and 50% lysis at an E:T ratio of 50:1 was obtained. The listeriolysin K^d peptide (GYKDGNEYI; SEQ ID NO: 18) and HIV D^d peptide

(RKSIRIQRGPGRAFVTIGKGKGKGY; SEQ ID NO: 19), used as negative controls, did not give rise to lysis (Fig. 3a).

Predicted H-2^d-restricted peptides (extracellular region MUC1)

A number of 9-mer peptides in the extracellular region are predicted to be CTL epitopes [(AVSMTSSVL(K^d), SEQ ID NO: 20; TTQGQDVTL(K^d), SEQ ID NO: 21; NAVSMTSSV(K^d), SEQ ID NO: 22; TSATQRSSV(K^d), SEQ ID NO: 23; SSTTQGQDV(K^d), SEQ ID NO: 24; SVPSSTEKN(D^d), SEQ ID NO: 25; EPASGSAAT(L^d), SEQ ID NO: 26; SPGSGSSTT(L^d), SEQ ID NO: 27; VPSSTEKNA(L^d), SEQ ID NO: 28; TPGGEKETS(L^d), SEQ ID NO: 29; TSATQRSSV(L^d), SEQ ID NO: 30; SSTTQGQDV(L^d), SEQ ID NO: 24] and were contained in peptide 33-103 (CTLp frequency=1/20,150) with scores of 58, 40, 29, 10, 10, 2.9, 39, 39, 36, 30, 10 and 10) respectively. A subset of these peptides were also contained in the 51-70 peptide(CTLp frequency=1/10,000) (Table 3). Of these, four were made (AVSMTSSVL(K^d), SEQ ID NO; 20, NAVSMTSSV(K^d), SEQ ID NO: 22; VPSSTEKNA(L^d), SEQ ID NO: 28; SVPSSTEKN (D^d), SEQ ID NO: 25) and tested. Three of the four peptides were indeed presented and one was not. The synthetic peptides AVSMTSSVL(K^d), SEQ ID NO: 20; NAVSMTSSV(K^d), SEQ ID NO: 22 and VPSSTEKNA(L^d), SEQ ID NO: 28 sensitised P815 target cells with 77%, 80% and 78% lysis at E:T of 50:1 respectively, while SVPSSTEKN (with the lowest predictive value) was inactive (Figure 3b). Therefore, AVSMTSSVL, SEQ ID NO: 20; VPSSTEKNA, SEQ ID NO: 28 and NAVSMTSSV, SEQ ID NO: 22 are CTL epitopes in peptides 33-103 and 51-70.

Predicted H-2^b restricted peptides.

Even though there were fewer identified peptide epitopes for C57BL/6 mice, there are a large number of potential CTL epitopes present in the peptides, albeit with low scores (Table 3). The 9-mer CRRKNYGQL (D^b, K^b), SEQ ID NO: 32 was contained in 471-493 (CTLp not detected) and had scores of 10 and 1.4. It weakly sensitised RMA targets to lysis by mannan-HMFG CTL with 20 % lysis at a E:T of 50:1 and 42% lysis at E:T of 100:1 (Figure 3c). The MUC1 VNTR peptides APGSTAPPA (D^b), SEQ ID NO: 33 and SAPDTRPAP (K^b), SEQ ID NO: 16 were used as positive specificity controls, where lysis of 70% and 80% were obtained while no lysis was detected for the ovalbumin K^b 9-mer SIINFEKL, SEQ ID NO: 11 and Adenovirus D^b 9-mer (used as negative specificity controls). The 9-mer peptides STEKNAVSM(D^b), SEQ ID NO: 34; AVSMTSSVL(D^b), SEQ ID NO: 20 and AVSMTSSVL(K^b),

SEQ ID NO: 20 were contained in the peptides 33-103 and 51-70 with scores of 15, 10 and 1.2. All of these three peptides weakly sensitised RMA targets to lysis (~20% at 50:1 and ~40% lysis at E:T of 100:1) (Figure 3d). There were no CTL reactive to peptides 31-55 and 51-70 in C57BL/6 mice.

Two high scoring CTL epitopes predicted from the whole MUC1 molecule from the intracellular region (YYQELQRDI(K^d), SEQ ID NO: 35 score 2880) and extracellular region N-terminal to the VNTR (SAPDNRPAL(D^b), SEQ ID NO: 36 score 4723) with scores of 2880 and 4723 sensitized RMA and P815 target cells to 50% lysis at an E:T of 50:1 (Figure 3e). Therefore, several T cell epitopes are present in the non-VNTR regions of the MUC1 molecule and 9-mer peptides can be presented by target cells to CTL generated by mannan-HMFG immunisation.

4. DISCUSSION

Previous immunisation studies by the present inventors used a MUC1 fusion protein containing 5 repeats of the VNTR linked to mannan (MFP) and this generated strong cellular responses to MUC1 characterised by the production of IFN-γ, IL-12, very little IgG_{2a} antibody and protection from tumour growth [36, 48]. Immune responses in humans have also shown promise for the therapeutic use of MUC1 antigens as in a Phase I clinical trial using MFP, 4 of 15 patients generated proliferative responses, 13 of 25 showed high levels of MUC1 specific serum antibody and 2 of 10 generated CTL to MUC1 [59]. However, *in vitro* peptide binding studies and *in vivo* studies using transgenic HLA-A*0201 mice demonstrated that the VNTR sequences can only be presented by HLA-A*0201 and HLA-A*1101 [39, 60], and studies thus far have concentrated on the MUC1 VNTR because of its preferential immunogenicity in mice, at least for antibodies, and because of evidence from humans implicating the VNTR in immune responses. Other protein sequences of MUC1 have not been examined for their cellular immunity. In the past, the present inventors have sought monoclonal antibodies to non-VNTR regions in mice immunised with MUC1: none resulted and none were found in an international study. Scanning the whole MUC1 sequence for potential T cell epitopes predicted many previously untested peptides. The inventors have therefore immunised mice with mannan conjugated HMFG, to provide all possible MUC1 epitopes but dependent on natural antigen processing for their presentation, and showed

that cellular immune responses to the non-VNTR regions of the MUC1 can be generated which are as effective as those generated to the VNTR and further both HLA-A*0201 and A2K^{b}MUC1 transgenic mice could be immunised, indicating that humans should also be able to be immunised.

Cellular responses could be detected to the extracellular region of MUC1, the VNTR and also to intracellular peptides in mannan-HMFG immunised BALB/c, C57BL/6, HLA-A*0201/K^{b} and double transgenic A2K^{b}MUC1 mice. Immunised BALB/c mice developed CTL that could respond to more non-VNTR CTL epitopes than C57BL/6 mice, in which only the 344-364 peptide and SAPDNRPAL (SEQ ID NO: 36) was recognised by CTL (Table 2, Figure 3e).

Of the various peptides used for restimulation, several possible candidate 9-mer epitopes could be predicted using the peptide motif search program (Table 3). In BALB/c mice, the precursor frequency for the 471-493 peptide was 1/12,500, however the predicted epitope peptides NYGQLDIFP (SEQ ID NO: 13), YGQLDIFPA (SEQ ID NO: 14) and KNYGQLDIF (SEQ ID NO: 15) were not able to sensitise P815 targets for lysis by mannan-HMFG CTL (Figure 3a). Therefore, either the stimulating CTL epitope was not correctly identified by the algorithm or these synthetic peptides were not appropriately processed and presented by the target cells. In contrast, several 9-mers present in the 33-103 and 51-70 sequences (AVSMTSSVL SEQ ID NO: 20; NAVSMTSSV, SEQ ID NO: 22 and VPSSTEKNA, SEQ ID NO: 28) were identified as functional CTL epitopes in the lysis assays (Figure 3b).

In C57BL/6 mice, the CRRKNYGQL, (SEQ ID NO: 32), STEKNAVSM (SEQ ID NO: 34) and AVSMTSSVL (SEQ ID NO: 20) peptides from the 51-70 and 471-493 sequences sensitised RMA cells for lysis however no CTLp were identified by restimulation with the larger peptides. This observation could result from the three 9-mers not being processed and presented by the MUC1^{+} cells.

Further analysis of the entire MUC1 sequence using the T cell epitope algorithm for mouse K^{d}, D^{d}, L^{d}, K^{b}, D^{b}, K^{k}, and human HLA-A1, HLA-A*0201, HLA-A3 and HLA-A24 epitopes show several candidate 9-mers for presentation by mouse or human cells. Of these 9-mer peptides, SAPDNRPAL (D^{b}), (SEQ ID NO: 36) and YYQELRDI (K^{d}) (SEQ ID NO: 35) were synthesised and both were very efficient in sensitising P815 or RMA cells for lysis by mannan-HMFG CTL (Figure 3e). It is apparent in this study and others that

the prediction of CTL epitopes is not always accurate. A comparison of the predicted and experimentally determined T cell epitopes for the VNTR region illustrates that the lower scores do not necessarily predict a lack of presentation or antigenicity (Table 4). For example, SAPDTRPAP (SEQ ID NO: 16) peptide has been confirmed to be a K^b-restricted epitope by class I stabilisation when incubated with the TAP defective RMA-S cells as well as by lysis of peptide pulsed RMA cells (Figure 3c), however the predicted score is only 0.004 ([38]). Similarly, the K^k, L^d and D^d was not predicted accurately [38]. The HLA-A*0201 T cell epitope, STAPPAHGV (SEQ ID NO: 37) identified independently by epitope mapping [39] was predicted albeit with a low score. The prediction algorithms act as a guide, to the probability of antigen presentation, but the *in vivo* response will be defined by antigen processing, immunodominance, T-cell repertoire, glycosylation and other unknown factors [61, 62].

The whole MUC1 protein in purified form has not previously been used to immunise mice to generate cellular immunity, although several other immunisation methods have been used. The whole MUC1 protein has been delivered in a vaccinia construct [46, 63], as a construct in DNA immunisation [64], in transfected dendritic cells [65] and in transfected EBV-B cells [66] . In none of these studies was the specificity of the CTL ascertained. However, the importance of using glycosylated MUC1 (as HMFG) should be stressed. Other studies, in mice and humans have used non-glycosylated peptides which have led to antibody production in both MUC1 transgenic mice [67] and in humans [59, 68, 69]; in these studies it was considered that B cell and at times T cell tolerance had been overcome but, with respect to antibodies, the non-glycosylated peptides represent novel antigens and the response is not surprising. However, in the studies described herein, native glycosylated mucin (HMFG) linked to mannan successfully primed CTL in several strains of mice including $A2K^bMUC1$ transgenic mice. Mannan-HMFG gave a higher CTLp frequency in $A2K^bMUC1$ mice (1/2000) compared to BALB/c or C57Bl/6 mice and could be due to either the different strain of mice or to the presence of a higher affinity HLA-A*0201 CTL epitope. In BALB/c mice, HMFG gave a CTLp frequency of 1/80,500. This was comparable to the CTLp frequency in mice immunised with a non-glycosylated form of MUC1 VNTR [47], i.e., both glycosylated and non-glycosylated forms of the VNTR were equally immunogenic provided they are

presented with oxidised mannan. Clearly, the carbohydrate coating did not obscure the underlying peptide. Thus, mannan-HMFG is able to break tolerance in A2K^{b}MUC1 transgenic mice by producing CTLs to peptides in the VNTR, the extracellular region and the intracellular region in MUC1. These results reinforce the concept that MUC1 should be a useful target in therapy.

The use of mannan-HMFG in humans warrants some discussion in that MUC1 is present on some normal cells such as pancreas, kidney. Hence, it is possible that immune responses may be generated to these tissues and give rise to autoimmunity. Thus far in our clinical trials using MUC1 VNTR conjugated to mannan no autoimmunity was detected, however, careful dose escalation studies and monitoring is necessary [59]. The HMFG obtained directly from donors is likely to be less preferred for use and recombinant material may be more appropriate. However, using recombinant material, the high level of glycosylation of the HMFG should be kept in mind. Presumably, a eukaryotic vector will be necessary. Thirdly, we have recently shown that the VNTR peptides can deviate the immune response towards antibodies, because of a cross reaction with existing, natural human antibodies [70]. Such a deviation may occur when using whole MUC1.

EXAMPLE 2

The non-VNTR peptides were coupled to keyhole limpet hemocyanin (KLH) using gluteraldehyde and then reacted with oxidised mannan as follows:

Two mg of the peptide 471 or 507 was dissolved in 1.75 ml phosphate buffer and mixed with 0.25 ml KLH (2mg/ml), treated with 1ml of 0.25% glutaraldehyde and allowed to mix in the dark overnight at room temperature. The mixture was dialysed into phosphate buffer overnight. The dialysed mixture was mixed with 1 ml oxidised mannan prepared as described in European Patent Application No. 94303817.4 and allowed to stand overnight.

BALB/c mice (6-8 weeks) were immunised intraperitoneally with 5 micrograms Mannan-peptide KLH on days 0, 10 and 17 and CTL activity in splenocytes determined as described. Non-VNTR peptide conjugated to mannan showed positive responses in the CTL assay (Figures 4 and 5) compared to the positive controls (VNTR peptides conjugated to mannan).

EXAMPLE 3

The non-leader, non-VNTR peptides and polypeptides may also be used for the preparation of DNA vaccines. This can be performed by using established procedures in DNA cloning and nucleic acid vaccination. For example, the nucleic acid sequence encoding one or more of the non-leader, non-VNTR peptides and polypeptides, with necessary restriction enzyme sites at the 3 and 5' ends can be synthesised in a automated DNA synthesiser and cloned into a suitable vector such as pcDNA3 or pSV3 [72]). The clones can be screened for incorporation of the nucleic acid sequences by restriction enzyme digests or protein expression. The DNA can then be injected into various sites in humans and other animals for immunisation.

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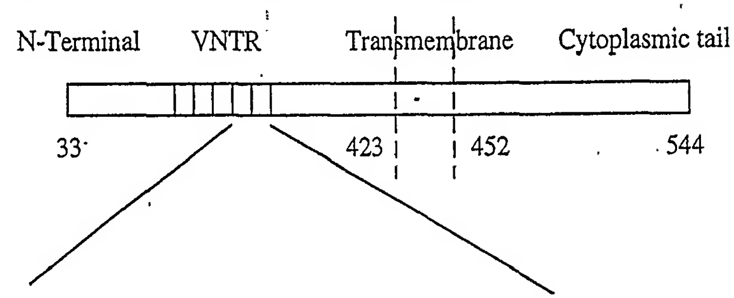
It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Table 1

Diagramatic Structure of MUC1 and Sequences of the Synthetic Peptides



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1 13 32

MUC1		
Extracellular region		
Cp13-32	(C)PAHGVTSAPDTRPAPGSTAP	(SEQ ID NO: 39)
p1-30	PDTRPAPGSTAPPAHGVTSAPDTRPAPGST	(SEQ ID NO: 40)
31-55	TGSGHASSTPGGEKETSATQRSSVP	(SEQ ID NO: 2)
51-70	RSSVPSSTEKNAVSMTSSVL	(SEQ ID NO: 3)
33-103	Glutathione -S-transferase fusion protein	
	SGHASSTPGGEKETSATQRSSVPSSTEKNAVSMTSSVLS	
	SHSPGSGSSTTQGQDVTLAPATEPASGSAATW	(SEQ ID NO: 4)
229-237	SAPDNRPAL	(SEQ ID NO: 6)
344-364	NSSLEDPSTDYYQELQRDISE	(SEQ ID NO: 7)
Intracellular region		
408-423	TQFNQYKTEAASRVNL	(SEQ ID NO: 8)
471-493	AVCQCRRKNYGQLDIFPARDTYH	(SEQ ID NO: 9)
507-526	(C)YVPPSSTDRSPYEKVSAGNG	(SEQ ID NO: 41)
Mouse CD4		
T4NI	KTLVLGKEQESAELPCECY	(SEQ ID NO: 42)

Table 2

CTLp Frequencies in Spleens of Mice Immunised With mannan-HMFG

Restimulating Antigen	Peptide Details	CTLp Frequency			
		Immunized Strain			
		C57BL6(K^bD^b)	BALB/c ($K^dD^dL^d$)	HLA*0201/K^b	A2K^bMUC1
		Target Cell			
		MUC1-RMA	Target MUC1-P815	EBV + pep	MCF7
Whole MUC1	HMFG	1/13,500	1/9,700	1/39,000	1/2,000
Extracellular region	Cp13-32	ND	1/7,000	ND	ND
	p1-30	1/12,500	1/13,200	1/33,000	1/8,000
	31-55	Not Detected	1/19,500	1/40,000	1/2,000
	51-70	Not Detected	1/10,000	ND	ND
	33-103	ND	1/20,150	ND	ND
	344-364	1/24,500	1/36,800	ND	1/11,000
Intracellular region	408-423	Not Detected	1/30,000	ND	1/20,000
	471-493	Not Detected	1/12,500	ND	Not detected
	507-526	Not Detected	1/22,500	ND	Not detected
Non-MUC1	T4N1	Not Detected	Not Detected	Not detected	Not Detected

Table 3

Mice Immunised with mannan-HMFG: CTLp Frequencies to Various non-VNTR Peptides and their Predicted CTL Epitopes

Peptide Used for Stimulation	CTLp Frequency		Predicted 9-mers and Score for the Various H2 Haplotypes									
	BALB/c	C57Bl/6	K^d	CTL	D^d	CTL	L^d	CTL	D^b	CTL	K^b	CTL
471-493 AVCQCRRKNYGQLDIFP ARDTYH (SEQ ID NO: 43)	1/12,500	not detected	NYGQLDIFP 6 (SEQ ID NO: 13)	0%	YGQLDIFPA 6 (SEQ ID NO: 14)	0%	KNYGQLDIF 10 (SEQ ID NO: 15)	0%	CRRKNYGQL 10 (SEQ ID NO: 32)	20%	CRRKNYGQL 1.4 (SEQ ID NO: 32)	20%
507-526 CYVPPSSTDRSPYEKVSA GNG (SEQ ID NO: 41)	1/22,500	not detected	CYVPPSSTD 12 (SEQ ID NO: 46)		VPPSSTDRS 2.4 (SEQ ID NO: 53)		VPPSSTDRS 45 (SEQ ID NO: 53)		RSPYEKVSA 0.99 (SEQ ID NO: 62)		VPPSSTDRS 0.6 (SEQ ID NO: 53)	
408-423 TGFNQYKTEAASRYNL (SEQ ID NO: 44)	1/30,000	not detected	QFNQYKTEA 29 (SEQ ID NO: 47) QYKTEAASR 6.0 (SEQ ID NO: 48) TEAASRVNL 5.8 (SEQ ID NO: 49)		FNQYKTEAA 2.2 (SEQ ID NO: 54)		TEAASRVNL 6.0 (SEQ ID NO: 57)		TEAASRVNL 0.18 (SEQ ID NO: 57)		FNQYKTEAA 0.72 (SEQ ID NO: 54)	
344-364 NSSLEDPSTDYYQELQRD ISE (SEQ ID NO: 7)	1/36,800	1/24,500	YYQELQRDI 2880 (SEQ ID NO: 35)		YYQELQRDI 1.8 (SEQ ID NO: 35)		NSSLEDPST 5 (SEQ ID NO: 58) DPSTDYYQE 3.6 (SEQ ID NO: 59) PSTDYYQEL 2.5 (SEQ ID NO: 60)		SSLEDPSTD 4.4 (SEQ ID NO: 63)		PSTDYYQEL 2.9 (SEQ ID NO: 60) YYQELQRDI 1.1 (SEQ ID NO: 35)	
			SLEDPSTDV 14 (SEQ ID NO: 50)									
33-103 SGHASSTPGGEKETSATQ RSSVPSSTEKNAVSMTSS VLSSHSPGSGSSTTQGQD VTLAPATEPASGSAATW (SEQ ID NO: 45)	1/20,150	not done	AVSMTSSVL 58 (SEQ ID NO: 20)	77%	SVPSSTEKN 2.9 (SEQ ID NO: 25)	0%	EPASGSAAT 39 (SEQ ID NO: 26)		STEKNAVSM 15 (SEQ ID NO: 34)	19%	AVSMTSSVL 1.2 (SEQ ID NO: 20)	18%

			TTQGQDVTL 40 (SEQ ID NO: 21)				SPGSGSSTT 39 (SEQ ID NO: 27)		AVSMTSSVL 10.1 (SEQ ID NO: 20)	18%		
			NAVSMTSV 29 (SEQ ID NO: 51)	80%			VPSSTEKNA 36 (SEQ ID NO:28)	78%				
			TSATQRSSV 10 (SEQ ID NO: 23)				TPGGEKETS 30 (SEQ ID NO: 29)					
			SSTTQGQDV 10 (SEQ ID NO: 31)				TSATORSSV 10 (SEQ ID NO: 23)					
							SSTTQGQDV 10 (SEQ ID NO: 31)					
51-70 RSSVPSSTEKNAVSMTSS VL (SEQ ID NO: 3)	1/10,000	not detected	AVSMTSSVL 58 (SEQ ID NO: 20)		SVPSSTEKN 2.9 (SEQ ID NO: 25)		VPSSTEKNA 36 (SEQ ID NO: 28)		STEKNAVSM 15 (SEQ ID NO: 34)		AVSMTSSVL 1.2 (SEQ ID NO: 20)	
			NAVSMTSV 29 (SEQ ID NO: 51)				AVSMTSSVL 5.0 (SEQ ID NO: 20)		AVSMTSSVL 10 (SEQ ID NO: 20)			
			STEKNVSM 6.0 (SEQ ID NO: 52)				SSTEKNAVS 5.0 (SEQ ID NO: 61)					
							NAVSMTSV 2.0 (SEQ ID NO: 51)					
31-55 TGSGHASSTPGGEKETSA TQRSSVP (SEQ ID NO: 2)	1/19,500	not detected	TSATQRSSV 10 (SEQ ID NO: 23)		GGEKETSAT 2.0 (SEQ ID NO: 55)		TPGGEKETS 30 (SEQ ID NO: 29)		TSATQRSSV 2.6 (SEQ ID NO: 23)		KETSATQRS 0.24 (SEQ ID NO: 64)	
					TGSGHASST 2.0 (SEQ ID NO: 56)		TSATQRSSV 10 (SEQ ID NO: 23)					

Table 4

Experimentally Determined and Predicted Mouse and Human CTL Epitopes in the MUC1 VNTR

Haplotype	Experimentally determined T cell epitope	Predicted Score for T cell epitope from algorithm	Predicted T cell epitopes	Predicted Score from algorithm
K^b	SAPDTRPAP (SEQ ID NO: 6)	0.004	APPAHGVTS (SEQ ID NO: 66)	0.330
			TAPPAHGVT (SEQ ID NO: 31)	0.300
K^d	ND		STAPPAHGV (SEQ ID NO: 37)	12.000
K^k	PDTRPAPGS (SEQ ID NO: 65)	0.200	STAPPAHGV (SEQ ID NO: 37)	0.5
L^d	APDTRPAPG (SEQ ID NO: 17)	0.900	APPAHGVTS (SEQ ID NO: 66)	45.00
			APGSTAPPA (SEQ ID NO:33)	30.00
D^d	SAPDTRPAP (SEQ ID NO: 6)	0.086	STAPPAHGV (SEQ ID NO: 37)	12.00
HLA-A*0201	STAPPAHGV (SEQ ID NO: 37)	0.966	STAPPAHGV (SEQ ID NO: 37)	0.966

Sequence Listing:

SEQUENCE LISTING

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35 40 45

Thr Glu Lys Asn Ala Val Ser Met Thr Ser Ser Val Leu Ser Ser His
50 55 60

Ser Pro Gly Ser Gly Ser Ser Thr Thr Gln Gly Gln Asp Val Thr Leu
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Ala Pro Ala Thr Glu Pro Ala Ser Gly Ser Ala Ala Thr Trp Gly Gln
85 90 95

Asp Val Thr Ser Val Thr Arg Pro Ala Leu Gly Ser Thr Thr Pro Pro
100 105 110

Ala His Asp Val Thr Ser Ala Pro Asp Asn Lys Pro Ala Pro Gly Ser
115 120 125

Thr Ala Pro Pro Ala His Gly Val Thr Ser Ala Pro Asp Thr Arg Pro
130 135 140

Ala Pro Gly Ser Thr Ala Pro Pro Ala His Gly Val Thr Ser Ala Pro
145 150 155 160

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Asp Asn Arg Pro Ala Leu Gly Ser Thr Ala Pro Pro Val His Asn Val
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Thr Ser Ala Ser Gly Ser Ala Ser Gly Ser Ala Ser Thr Leu Val His
            180                 185                 190

Asn Gly Thr Ser Ala Arg Ala Thr Thr Thr Pro Ala Ser Lys Ser Thr
        195                 200                 205

Pro Phe Ser Ile Pro Ser His His Ser Asp Thr Pro Thr Thr Leu Ala
    210                 215                 220

Ser His Ser Thr Lys Thr Asp Ala Ser Ser Thr His His Ser Thr Val
225                 230                 235                 240

Pro Pro Leu Thr Ser Ser Asn His Ser Thr Ser Pro Gln Leu Ser Thr
                245                 250                 255

Gly Val Ser Phe Phe Phe Leu Ser Phe His Ile Ser Asn Leu Gln Phe
            260                 265                 270

Asn Ser Ser Leu Glu Asp Pro Ser Thr Asp Tyr Tyr Gln Glu Leu Gln
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305                 310                 315                 320

Leu Thr Leu Ala Phe Arg Glu Gly Thr Ile Asn Val His Asp Val Glu
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Thr Ile Ser Asp Val Ser Val Ser Asp Val Pro Phe Pro Phe Ser Ala
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Claims:

1. A peptide or polypeptide capable of eliciting an immune response, wherein said peptide or polypeptide comprises an amino acid sequence substantially corresponding to that of an epitope of the non-VNTR, non-leader region of a mucin.

2. A peptide or polypeptide according to claim 1, wherein said peptide or polypeptide consists of an amino acid sequence derived from the non-VNTR, non-leader region of a mucin.

3. A peptide or polypeptide according to claim 1, wherein said epitope is from an extracellular region of the non-VNTR, non-leader region of a mucin.

4. A peptide or polypeptide according to claim 1, wherein said epitope is from an intracellular region of the non-VNTR, non-leader region of a mucin.

5. A peptide or polypeptide according to claim 1, wherein said epitope is from a transmembrane region of the non-VNTR, non-leader region of a mucin.

6. A peptide or polypeptide according to any one of the preceding claims, wherein said mucin is mucin 1 (MUC1).

7. A peptide or polypeptide according to claim 6, wherein said mucin 1 is human mucin 1.

8. A peptide or polypeptide according to claim 7, wherein said human mucin 1 is human milk fat globule membrane antigen (HMFG).

9. A peptide or polypeptide according to claim 3, wherein said epitope has an amino acid sequence selected from: AVSMTSSVL (SEQ ID NO: 20), NAVSMTSSV (SEQ ID NO: 22), VPSSTEKNA (SEQ ID NO: 28) and SAPDNRPAL (SEQ ID NO: 36).

10. A peptide or polypeptide according to claim 4, wherein said epitope has the amino acid sequence: YYQELQRDI (SEQ ID NO: 35).

11. A peptide or polypeptide according to claim 1 or 2, wherein said peptide or polypeptide comprises an amino acid sequence substantially corresponding to one of the following amino acid sequences or an immunogenic fragment thereof: TGSGHASSTPGGEKETSATQRSSVP (SEQ ID NO: 2), RSSVPSSTEKNAVSMTSSVL (SEQ ID NO: 3), SGHASSTPGGEKETSATQRSSVPSSTEKNAVSMTSSVLSSHSPGSGSSTTQG QDVTLAPATEPASGSAATW (SEQ ID NO: 4), SAPDNRPAL (SEQ ID NO: 6), NSSLEDPSTDYYQELQRDISE (SEQ ID NO: 7), TQFNQYKTEAASRVNL (SEQ ID NO: 8), AVCQCRRKNYGQLDIFPARDTYH (SEQ ID NO: 9) and YVPPSSTDRSPYEKVSAGNG (SEQ ID NO: 10).

12. A fusion protein comprising a peptide or polypeptide according to any one of the preceding claims with a suitable carrier protein.

13. A fusion protein according to claim 12, wherein said fusion protein is conjugated to a carbohydrate polymer.

14. A fusion protein according to claim 13, wherein the carbohydrate polymer is a polymer of a carbohydrate monomer unit selected from the group consisting of glucose, galactose, mannose, xylose, arabinose, fucose, glucosamine, galactosamine, rhamnose, 6-0-methyl1-D-galactose, 2-0-acetyl-β-D-xylose, N-acetyl-glucosamine, iduronate, guluronate, mannuronate, methyl galacturonate, α-D-galactopyranose 6-sulphate, fructose, α abequose and conformation and configuration isomers thereof, or is a polymer of a carbohydrate formed of two or more different types of said carbohydrate monomer units.

15. A fusion protein according to claim 14, wherein said carbohydrate polymer comprises at least 20 monomer units.

16. A fusion protein according to claim 15, wherein said carbohydrate polymer comprises more than 1000 monomer units.

17. A fusion protein according to claim 16, wherein said carbohydrate polymer comprises more than 10,000 monomer units.

18. A fusion protein according to any one of claims 13-17, wherein the carbohydrate polymer is a polymer of mannose or is a carbohydrate polymer comprising mannose.

19. A fusion protein according to any one of claims 13-17, wherein the carbohydrate polymer is a polymer of oxidised mannose or is oxidised mannan.

20. A peptide or polypeptide according to any one of claims 1-11 coupled to a suitable carrier protein.

21. A peptide or polypeptide according to claim 20, wherein said peptide or polypeptide and/or said carrier protein is conjugated to a carbohydrate polymer.

22. A peptide or polypeptide according to claim 21, wherein the carbohydrate polymer is a polymer of a carbohydrate monomer unit selected from the group consisting of glucose, galactose, mannose, xylose, arabinose, fucose, glucosamine, galactosamine, rhamnose, 6-0-methyl1-D-galactose, 2-0-acetyl-β-D-xylose, N-acetyl-glucosamine, iduronate, guluronate, mannuronate, methyl galacturonate, α-D-galactopyranose 6-sulphate, fructose, α abequose and conformation and configuration isomers thereof, or is a polymer of a carbohydrate formed of two or more different types of said carbohydrate monomer units.

23. A peptide or polypeptide according to claim 22, wherein said carbohydrate polymer comprises at least 20 monomer units.

24. A peptide or polypeptide according to claim 23, wherein said carbohydrate polymer comprises more than 1000 monomer units.

25. A peptide or polypeptide according to claim 24, wherein said carbohydrate polymer comprises more than 10,000 monomer units.

26. A peptide or polypeptide according to any one of claims 21-25, wherein the carbohydrate polymer is a polymer of mannose or is a carbohydrate polymer comprising mannose.

27. A peptide or polypeptide according to any one of claims 21-25, wherein the carbohydrate polymer is a polymer of oxidised mannose or is oxidised mannan.

28. A compound comprising a conjugate of a peptide or polypeptide according to any one of claims 1-11 and a carbohydrate polymer.

29. A compound comprising a conjugate of mucin 1 and a carbohydrate polymer, such that the conjugate is capable of eliciting a cell mediated immune response in a human or other animal.

30. A compound according to claim 29, wherein said mucin 1 is human mucin 1.

31. A compound according to claim 30, wherein said human mucin 1 is human milk fat globule membrane antigen (HMFG).

32. A compound according to any one of claims 28-31, wherein the carbohydrate polymer is a polymer of a carbohydrate monomer unit selected from the group consisting of glucose, galactose, mannose, xylose, arabinose, fucose, glucosamine, galactosamine, rhamnose, 6-0-methyl1-D-galactose, 2-0-acetyl-β-D-xylose, N-acetyl-glucosamine, iduronate, guluronate, mannuronate, methyl galacturonate, α-D-galactopyranose 6-sulphate, fructose, α abequose and conformation and configuration isomers thereof, or is a polymer of a carbohydrate formed of two or more different types of said carbohydrate monomer units.

33. A compound according to claim 32, wherein said carbohydrate polymer comprises at least 20 monomer units.

34. A compound according to claim 33, wherein said carbohydrate polymer comprises more than 1000 monomer units.

35. A compound according to claim 34, wherein said carbohydrate polymer comprises more than 10,000 monomer units.

36. A compound according to any one of claims 28-35, wherein the carbohydrate polymer is a polymer of mannose or is a carbohydrate polymer comprising mannose.

37. A compound according to any one of claims 28-35, wherein the carbohydrate polymer is a polymer of oxidised mannose or is oxidised mannan.

38. A vaccine or therapeutic agent comprising a peptide or polypeptide according to any one of claims 1-11 or 20-27 or a fusion protein according to any one of claims 12-19 and, optionally, an adjuvant and/or a pharmaceutically acceptable carrier.

39. A vaccine or therapeutic agent comprising a conjugate compound according to any one of claims 28-37 and, optionally, an adjuvant and/or a pharmaceutically acceptable carrier.

40. A method for inducing a cell mediated immune response against mucin which comprises administering to a subject an effective amount of a peptide or polypeptide according to any one of claims 1-11 or 20-27 or a fusion protein according to any one of claims 12-19, optionally in combination with an adjuvant and/or a pharmaceutically acceptable carrier.

41. A method for inducing a cell mediated immune response against mucin which comprises administering to a subject an effective amount of a conjugate compound according to any one of claims 28-37, optionally in combination with an adjuvant and/or a pharmaceutically acceptable carrier.

42. A method of preventing or treating a carcinoma in a subject, said method comprising administering to said subject a vaccine or therapeutic agent according to claim 38 or 39.

43. A method according to claim 42, wherein said carcinoma is an adenocarcinoma.

44. A method according to claim 43, wherein said adenocarcinoma is breast cancer.

45. The use of a peptide or polypeptide according to any one of claims 1-11 or 20-27, a fusion protein according to any one of claims 12-19, or a conjugate compound according to any one of claims 28-37, to pulse dendritic cells for *in vivo* transfer and use as a vaccine.

46. An isolated nucleic acid molecule comprising a nucleotide sequence encoding the peptide or polypeptide of any one of claims 1-11 or a fusion protein according to any one of claims 12-19.

47. A DNA vaccine comprising a nucleic acid molecule according to claim 46.

Figure 1

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DNKPAPGSTA P**PAHGVTSAP DTRPAPGSTA PPAHGVTSAP DNRPALGSTA** PPVHNVTSAS 180

GSASGSASTL VHNGTSARAT TTPASKSTPF SIPSHHSDTP TTLASHSTKT DASSTHHSTV 240
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DVPFPFSAQS GAGVPGWGIA LLVLVCVLVA LAIVYLIALA VCQCRRKNYG QLDIFPARDT 420

YHPMSEYPTY HTHGRYVPPS STDRSPYEKV SAGNGGSSLS YTNPAVAATS ANL 473

- leader sequence is shown in italics
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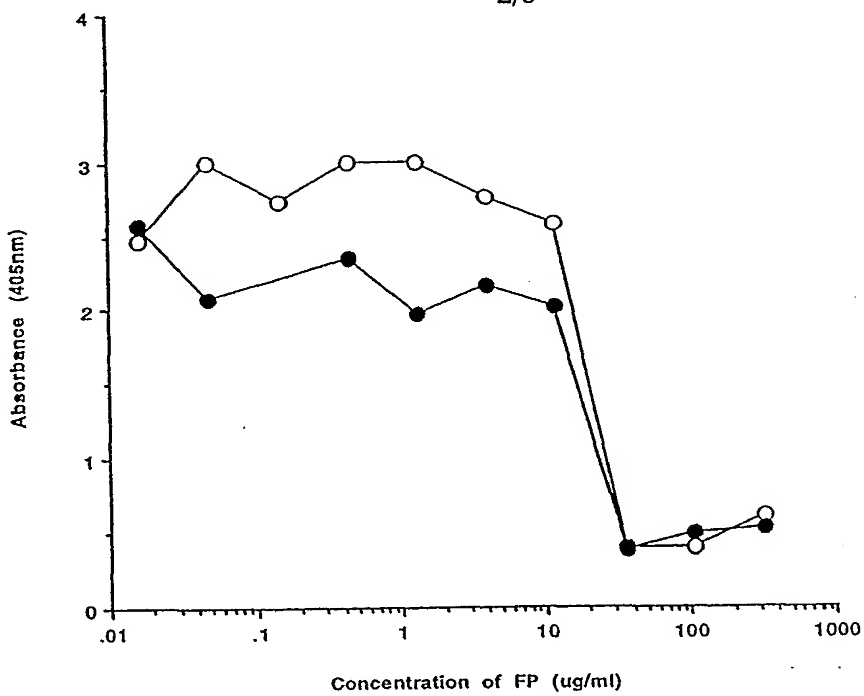

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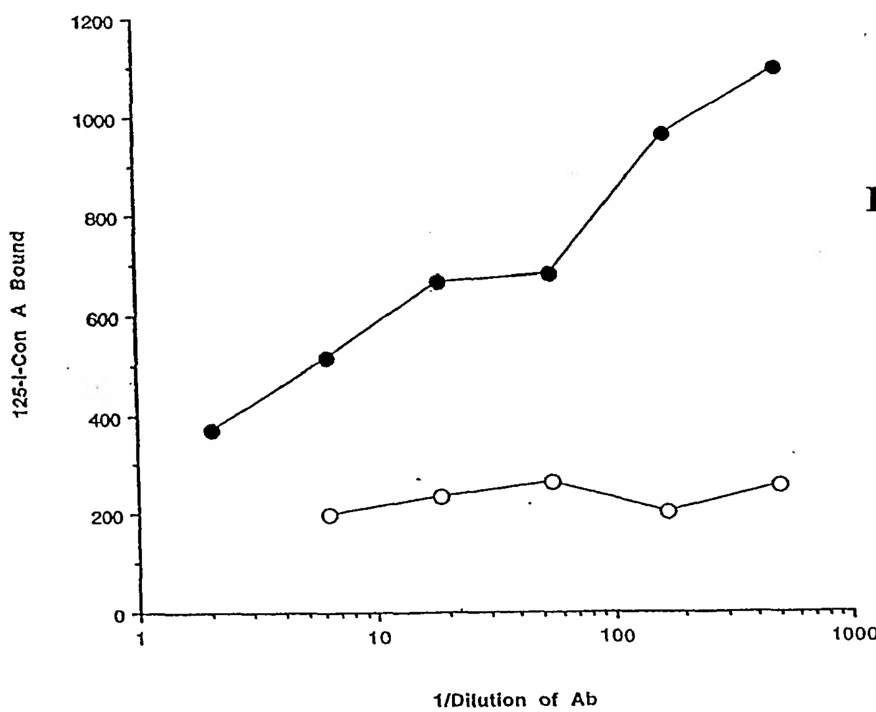


FIGURE 2B

FIGURE 3

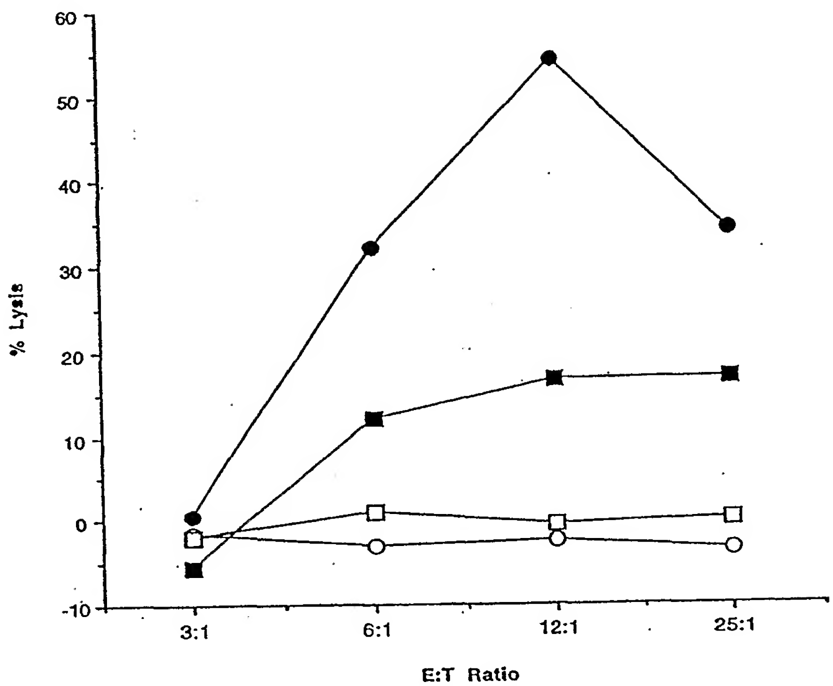
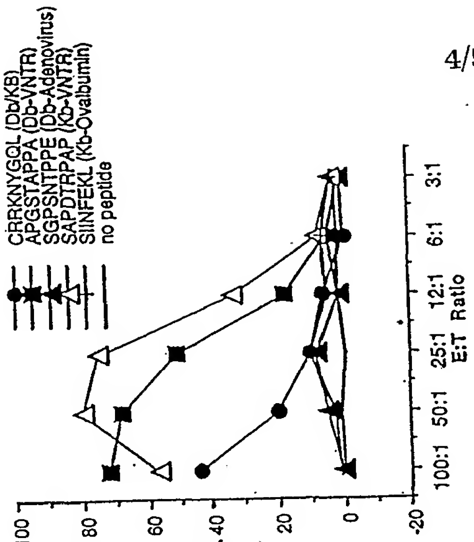


FIGURE 4C



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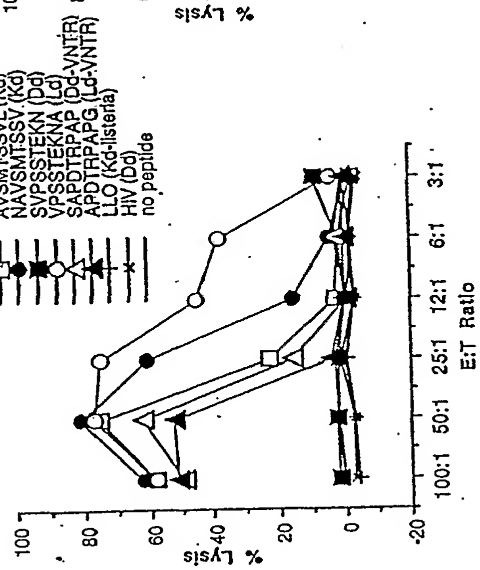

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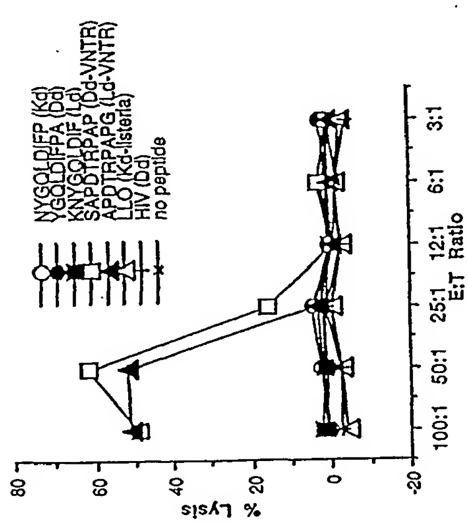

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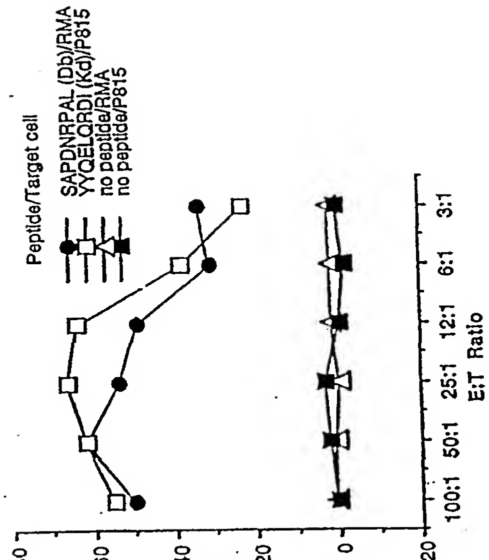

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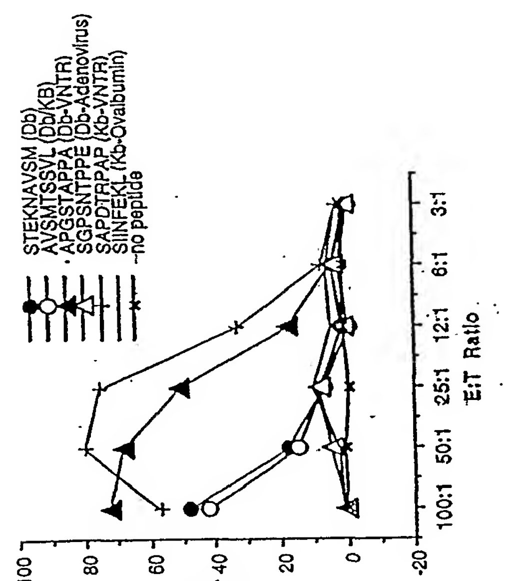

CTL Activity in Mice Immunised with Mannan-507-KLH

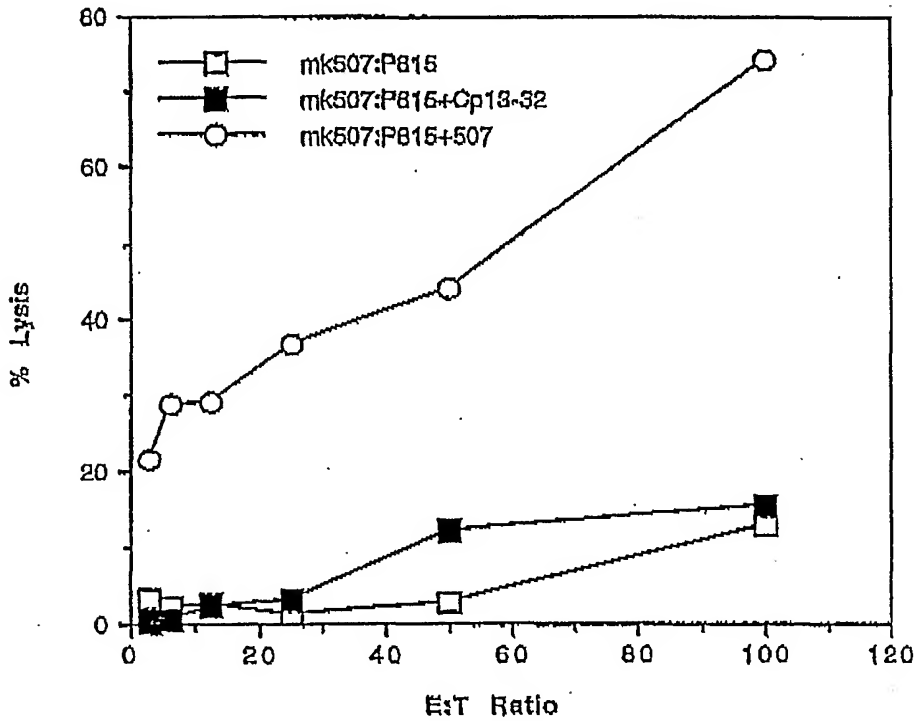

FIGURE 5

CTL Activity in Mice immunised with Mannan-471-KLH

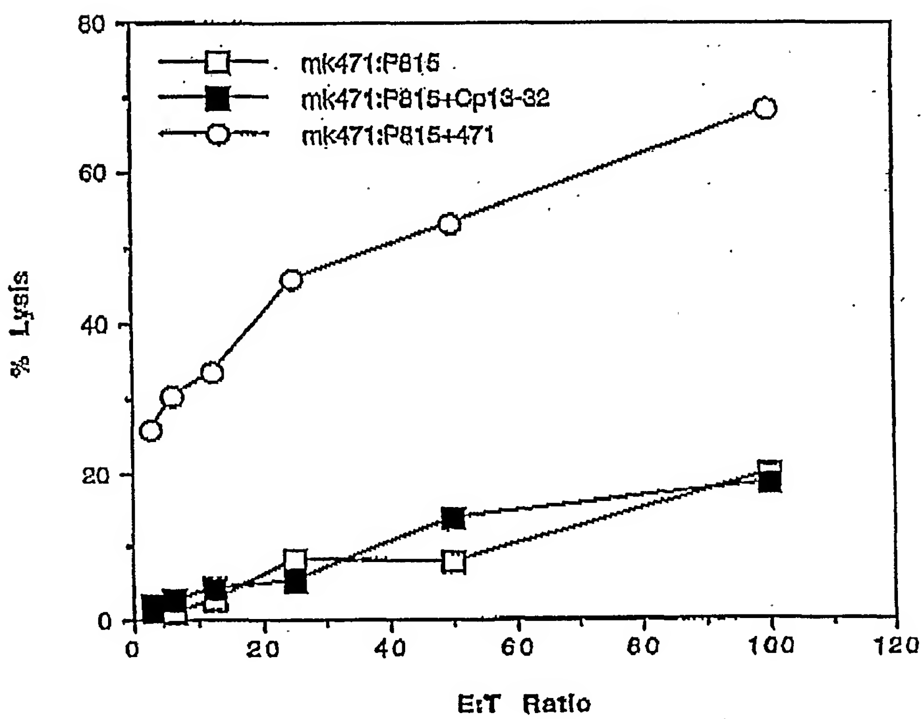

FIGURE 6

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Ser His Ser Thr Lys Thr Asp Ala Ser Ser Thr His His Ser Thr Val
225                 230                 235                 240

Pro Pro Leu Thr Ser Ser Asn His Ser Thr Ser Pro Gln Leu Ser Thr
                245                 250                 255

Gly Val Ser Phe Phe Phe Leu Ser Phe His Ile Ser Asn Leu Gln Phe
            260                 265                 270

Asn Ser Ser Leu Glu Asp Pro Ser Thr Asp Tyr Tyr Gln Glu Leu Gln
        275                 280                 285

Arg Asp Ile Ser Glu Met Phe Leu Gln Ile Tyr Lys Gln Gly Gly Phe
    290                 295                 300

Leu Gly Leu Ser Asn Ile Lys Phe Arg Pro Gly Ser Val Val Val Gln
305                 310                 315                 320

Leu Thr Leu Ala Phe Arg Glu Gly Thr Ile Asn Val His Asp Val Glu
                325                 330                 335

Thr Gln Phe Asn Gln Tyr Lys Thr Glu Ala Ala Ser Arg Tyr Asn Leu
            340                 345                 350

Thr Ile Ser Asp Val Ser Val Ser Asp Val Pro Phe Pro Phe Ser Ala
        355                 360                 365

Gln Ser Gly Ala Gly Val Pro Gly Trp Gly Ile Ala Leu Leu Val Leu
    370                 375                 380

Val Cys Val Leu Val Ala Leu Ala Ile Val Tyr Leu Ile Ala Leu Ala
385                 390                 395                 400

Val Cys Gln Cys Arg Arg Lys Asn Tyr Gly Gln Leu Asp Ile Phe Pro
                405                 410                 415

Ala Arg Asp Thr Tyr His Pro Met Ser Glu Tyr Pro Thr Tyr His Thr
            420                 425                 430

His Gly Arg Tyr Val Pro Pro Ser Ser Thr Asp Arg Ser Pro Tyr Glu
        435                 440                 445

Lys Val Ser Ala Gly Asn Gly Gly Ser Ser Leu Ser Tyr Thr Asn Pro
    450                 455                 460

Ala Val Ala Ala Thr Ser Ala Asn Leu
465                 470


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Arg Asp Ile Ser Glu
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Ala Gly Asn Gly
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Ile Gly Lys Gly Lys Gly Lys Gly Tyr
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Gly Ser Thr Ala Pro
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Ser Ala Gly Asn Gly
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Glu Cys Tyr

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Pro Ala Arg Asp Thr Tyr His
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Met Thr Ser Ser Val Leu Ser Ser His Ser Pro Gly Ser Gly Ser Ser
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Ser Gly Ser Ala Ala Thr Trp
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